

Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn

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Using the blind whole cell patch-clamp recording technique, we investigated peripheral nerve injury-induced changes in excitatory synaptic transmission to neurones in lamina II of the dorsal horn. Partial (i.e. chronic constriction injury (CCI) and spared nerve injury (SNI)) and complete (i.e. sciatic nerve transection (SNT)) peripheral nerve injury altered the mean threshold intensity for eliciting A fibre-mediated EPSCs in lamina II neurones. Following SNT and CCI, EPSC threshold was significantly decreased, but following SNI, EPSC threshold was increased (naive: $32 \pm 2 \mu\text{A}$, SNT: $22 \pm 2 \mu\text{A}$, CCI: $23 \pm 2 \mu\text{A}$, SNI: $49 \pm 4 \mu\text{A}$; $P < 0.01$, Student's unpaired *t* test). Despite this disparity between models, dorsal root compound action potential recordings revealed no significant difference in the conduction velocity or activation threshold of A β and A δ fibres in naive, SNT, CCI and SNI rats. In addition to the changes in EPSC threshold, we also observed a shift in the distribution of EPSCs. In spinal cord slices from naive rats, polysynaptic A β fibre-evoked EPSCs were observed in 24 % of lamina II neurones, monosynaptic A δ fibre EPSCs were observed in 34 % and polysynaptic A δ fibre EPSCs were observed in 7 %. Following SNT and CCI, the percentage of neurones with polysynaptic A β fibre EPSCs increased to ≥ 65 % of the sampled population, while the percentage of neurones with monosynaptic A δ fibre EPSCs decreased to < 10 %. The percentage of neurones with polysynaptic A δ fibre EPSCs was unchanged. In contrast, following SNI, A β fibre EPSCs decreased in incidence while the percentage of neurones with polysynaptic A δ fibre EPSCs increased to 44 %. Similar to the other injury models, however, monosynaptic A δ fibre EPSCs decreased in frequency following SNI. Thus, excitatory synaptic transmission is subject to divergent plasticity in different peripheral nerve injury models, reflecting the complexity of responses to different forms of deafferentation.

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In naive animals, there is a distinct laminar termination of primary afferent neurones in the spinal dorsal horn, such that lamina II (substantia gelatinosa; SG) receives predominantly high-threshold inputs from thinly myelinated A δ and unmyelinated C primary afferent fibres (Willis & Coggeshall, 1991). Consequently, electrophysiological recordings from SG neurones in adult spinal cord slices reveal a characteristic distribution of EPSCs in response to dorsal root stimulation (Baba *et al.* 1999). A β fibre-evoked polysynaptic EPSCs are observed in only a very small proportion of SG neurones, while, mono- and/or polysynaptic A δ fibre-mediated EPSCs are detectable in the vast majority (Yoshimura & Nishi, 1993; Baba *et al.* 1999).

Following complete peripheral nerve injury, A β fibres sprout from their normal termination site in the deep dorsal horn into the SG (Woolf *et al.* 1992, 1995; Koerber *et al.* 1994). Though some controversy exists regarding the

extent and pattern of such central sprouting (Bao *et al.* 2002), novel mono- (Kohama *et al.* 2000) or polysynaptic (Okamoto *et al.* 2001) A β fibre-mediated EPSCs begin to be observed in SG neurones following a sciatic nerve transection. Anatomical changes compatible with A fibre sprouting have also been reported following chronic constriction injury (CCI) of the sciatic nerve (Nakamura & Myers, 1999), a partial nerve injury model where some intact peripheral innervation and input to the dorsal horn remains.

In addition to central sprouting, peripheral nerve injury is associated with atrophic changes in central afferent terminals (Kapadia & LaMotte, 1987), which might alter excitatory synaptic transmission. Changes also occur in intrinsic dorsal horn neurones, for example, partial (spared nerve injury (SNI) and CCI), but not complete, sciatic nerve injury results in a selective loss of GABA_A-

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mediated IPSCs in lamina II neurones (Moore *et al.* 2002). Alterations in excitatory synaptic transmission produced by partial peripheral nerve injury have not yet been examined. We have now investigated excitatory synaptic responses in SG neurones following two types of partial sciatic nerve injury, CCI and SNI, and after a complete sciatic nerve transection (SNT), to assess excitatory synaptic drive following these different forms of deafferentation.

METHODS

Peripheral nerve injury models

As approved by our Institutional Animal Care and Use Committee, SNT, CCI or SNI was performed on the left sciatic nerve of adult male Sprague Dawley rats (5–6 weeks) under halothane (2.5%) anaesthesia. For SNT, the sciatic nerve was ligated and severed in the popliteal fossa. For CCI, four 4-0 chromic gut sutures spaced ~1 mm apart were loosely tied around the sciatic nerve proximal to the trifurcation (Bennett & Xie,

1988). For SNI, the common peroneal and tibial nerve branches of the sciatic nerve were tightly ligated with 5-0 silk and sectioned distal to the ligation (Decosterd & Woolf, 2000). All CCI and SNI rats developed mechanical and cold allodynia within 4 days of injury which persisted for > 2 weeks post injury.

Spinal cord slice preparation and electrophysiological recording

Spinal cords were removed from naive and nerve-injured rats under urethane anaesthesia (1.5 g kg⁻¹, i.p.). Thick (600–650 μ m) spinal cord slices with the L4 dorsal root (10–20 mm) left intact were prepared from naive adult rats (7–8 weeks) and rats subjected to nerve injury (SNT or CCI or SNI, 7–8 weeks, 2 weeks post injury) as described previously (Yoshimura & Nishi, 1993) (Fig. 1A). Following preparation, slices were perfused with oxygenated Krebs solution (10 ml min⁻¹; 36 \pm 1°C; composition (mM): NaCl 117, KCl 3.6, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25 and D-glucose 11) in the recording chamber for at least 30 min prior to recording. Whole cell patch pipettes were fabricated from borosilicate glass capillaries (1.5 mm o.d., World Precision Instruments Inc.) and averaged 5–10 M Ω when filled

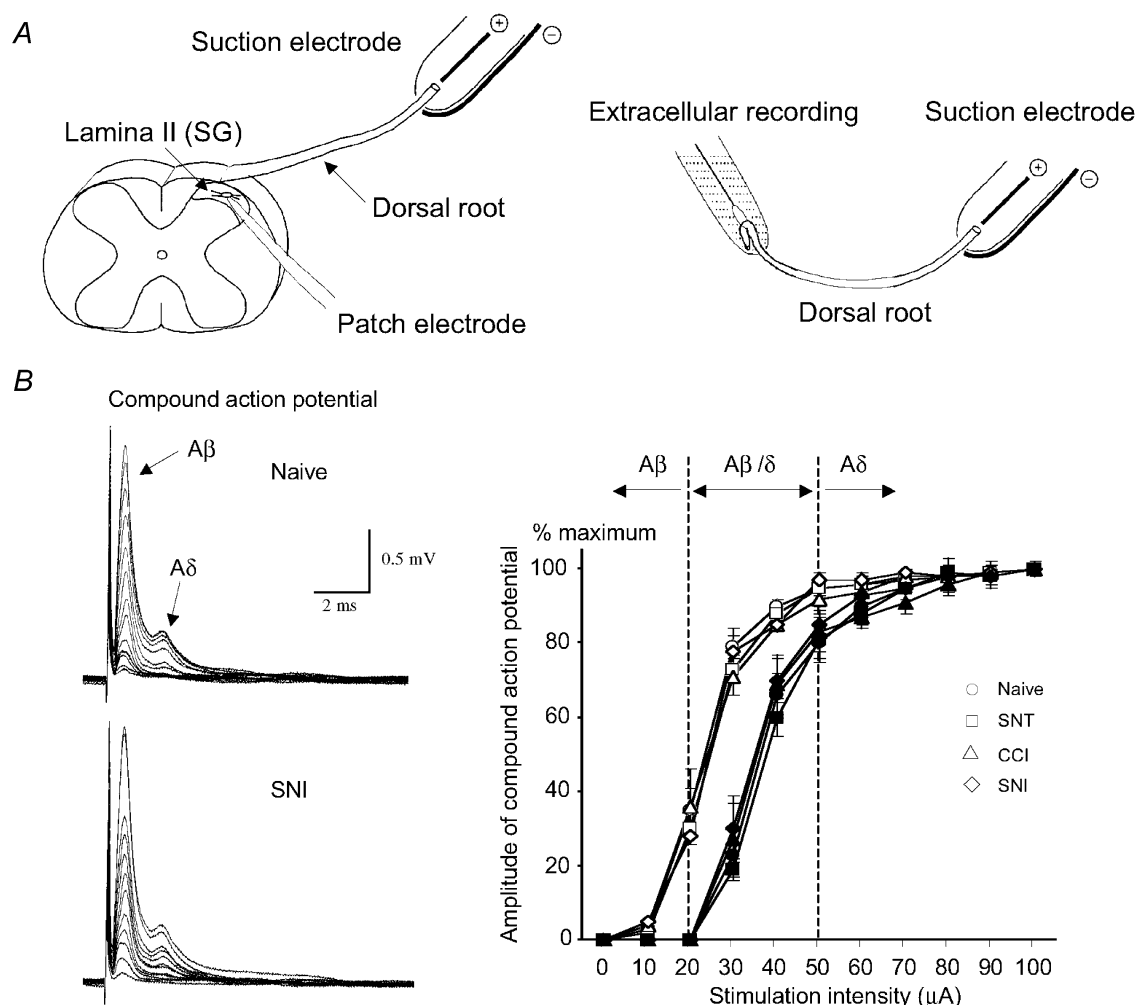


Figure 1. Dorsal root compound action potential recording

A, schematic diagram of our configuration for whole cell recording of EPSCs and dorsal root recording of compound action potentials. B, representative graded intensity A fibre-evoked compound action potentials recorded extracellularly from naive and SNI rats are shown on the left. A summary of the A β and A δ fibre stimulus–response relationships recorded in naive and nerve-injured rats is shown on the right ($n = 5–8$). Open symbols represent A β fibre responses, filled symbols represent A δ fibre responses.

Table 1. Compound action potentials

	Threshold (μA)			Conduction velocity (m s^{-1})		
	$A\beta$ (0.05 ms)	$A\delta$ (0.05 ms)	C (0.5 ms)	$A\beta$	$A\delta$	C
Naive ($n=13$)	15 ± 1	26 ± 1	262 ± 23	25.8 ± 1.2	7.9 ± 0.3	0.8 ± 0.1
SNT ($n=5$)	14 ± 1	27 ± 1	225 ± 21	24.7 ± 1.1	8.0 ± 0.5	0.7 ± 0.2
CCI ($n=10$)	15 ± 1	25 ± 1	245 ± 19	24.7 ± 1.1	7.8 ± 0.4	0.9 ± 0.1
SNI ($n=8$)	13 ± 2	26 ± 3	$140 \pm 9^*$	26.9 ± 2.0	8.8 ± 0.5	1.0 ± 0.1

Values are means \pm S.E.M. * $P < 0.05$ compared to naive.

with internal solution (mM): Cs_2SO_4 110, CaCl_2 0.5, MgCl_2 2, TEA-Cl 5, ATP-Mg salt 5, EGTA 5 and Hepes 5). Membrane currents were amplified with an Axopatch 200A (Axon Instruments). Signals were filtered at 2 kHz and digitised at 5 kHz. Data was collected using pCLAMP6.3 or 8.0 and analysed using pCLAMP and Mini-analysis software (Synaptosoft, Inc., Decatur, USA).

In the case of the SNI model, neurones in the medial SG of spinal segment L4 will receive input from the damaged tibial and common peroneal nerves while the lateral SG will have predominantly undamaged sural nerve inputs. In the CCI model, the input to the medial and lateral SG at L4 will be a combination of injured and uninjured afferents, while after SNT input to both the medial and lateral SG will be from damaged afferents. Spontaneous and synaptically evoked fast EPSCs were recorded only from medial SG neurones (which receive input from injured fibres in all three of the injury models) voltage clamped to -70 mV (Yoshimura & Nishi, 1993; Kohno *et al.* 1999). Synaptically evoked currents were elicited by graded intensity dorsal root stimulation sufficient to recruit $A\beta$, $A\delta$ and C fibres. Classification of synaptic responses into $A\beta$, $A\delta$ and C fibres was based on a combination of response threshold and conduction velocity. A fibre EPSCs were classified as monosynaptic if response latency remained constant and there was an absence of failures upon high-frequency (20 Hz) stimulation (Baba *et al.* 1999). Identification of monosynaptic C fibre-mediated EPSCs was based on an absence of failures with low frequency (1 Hz) stimulation (Ataka *et al.* 2000; Nakatsuka *et al.* 2000).

To determine primary afferent response thresholds and conduction velocities under our recording conditions, the dorsal root was stimulated orthodromically (0.05 ms for A fibres, 0.5 ms for C fibres) and compound action potentials were recorded extracellularly near the dorsal root entry zone (Fig. 1A). The A and C fibre conduction velocities observed in the current study are similar to those measured previously from compound action potential recordings (Baba *et al.* 1999; Kohama *et al.* 2000) and intracellular recordings from dorsal root ganglion neurones (Ataka *et al.* 2000; Nakatsuka *et al.* 2000).

Data analysis

Numerical data are expressed as means \pm S.E.M. Statistical differences between naive and SNT, CCI or SNI rats were assessed using Student's unpaired *t* test. The *z* test was used to compare differences in percentage of neurones with a given type of EPSC. $P < 0.05$ was considered significant.

RESULTS

Primary afferent threshold and conduction velocity are similar in the dorsal roots of naive and nerve-injured rats

Primary afferent fibres can be divided into three distinct

groups, $A\beta$, $A\delta$ and C, based on response threshold and conduction velocity of extracellularly recorded compound action potentials (Table 1). The A fibre stimulus-response relationship observed in the current study (Fig. 1B) is similar to that shown previously in our laboratory (Baba *et al.* 1999). At $< 20 \mu\text{A}$ (0.05 ms), only $A\beta$ fibres are activated. Above $20 \mu\text{A}$ (0.05 ms) $A\delta$ fibres begin to be recruited. The $A\beta$ fibre volley reaches a maximum at $50 \mu\text{A}$; therefore any new response recruited above $50 \mu\text{A}$ is mediated by recruitment of $A\delta$ fibres. It is not possible, however, to differentiate $A\beta$ and $A\delta$ responses between 20 and $50 \mu\text{A}$ (0.05 ms). To activate C fibres from naive rats, a stimulus $> 200 \mu\text{A}$ is required with a pulse width of 0.5 ms.

In agreement with earlier *in vivo* (Villiere & McLachlan, 1996) and *in vitro* (Baba *et al.* 1999) reports, the average conduction velocities of $A\beta$, $A\delta$ and C fibres from naive rats are 25.8 ± 1.2 and 7.9 ± 0.3 , $0.8 \pm 0.1 \text{ m s}^{-1}$, respectively (Table 1), and are similar for naive, SNT, CCI and SNI rats (Table 1). The response thresholds for activating $A\beta$ and $A\delta$ fibres do not change after any of the nerve lesions, but the activation threshold of C fibres is significantly reduced following SNI (Table 1).

Membrane properties and spontaneous excitatory synaptic responses of SG neurones are unaffected by peripheral nerve injury

The membrane potential of SG neurones from naive rats is $-62 \pm 1 \text{ mV}$ ($n = 60$) and does not change following any of the nerve injuries (see Moore *et al.* 2002). The mean amplitude and frequency of spontaneous EPSCs are also unaffected by either partial or complete nerve injury (Fig. 2).

The distribution of excitatory synaptic responses in SG neurones is altered following peripheral nerve injury

All SG neurones respond to dorsal root stimulation with EPSCs ($n = 83$ naive neurones; $n = 29$ SNT neurones; $n = 57$ CCI neurones and $n = 43$ SNI neurones, Fig. 3A). EPSCs that reliably follow high-frequency repetitive stimulation with a constant latency are considered monosynaptic, while EPSCs that display variable latencies and failures are considered polysynaptic (Fig. 3B).

EPSCs can be divided into different groups based upon response thresholds and latency ($A\beta$, $A\delta$ and C), as well as responses to repetitive stimulation (putative mono- and

polysynaptic) (Fig. 3B). Due to the stimulus response profile of the afferent volleys, it is not possible to distinguish between $A\beta$ and $A\delta$ responses at stimulus thresholds between ~ 25 and $50 \mu\text{A}$, therefore we classified responses between these stimulus intensities as $A\beta/\delta$ (Fig. 3C). Most (57 out of 83; 69 %) SG neurones recorded from naive rats exhibit either polysynaptic $A\beta/\delta$ or monosynaptic $A\delta$ fibre-mediated EPSCs in response to dorsal root stimulation (Fig. 3C). A small proportion of naive neurones (20 out of 83; 24 %) display polysynaptic $A\beta$ fibre-mediated EPSCs (Fig. 3C), in agreement with earlier findings (Yoshimura & Nishi, 1993; Baba *et al.* 1999), and one-third of the naive neurones (25 out of 83; 30 %) display monosynaptic C fibre-evoked EPSCs.

In contrast, the majority of SG neurones from SNT (21 out of 29; 72 %) and CCI (37 out of 57; 65 %) slices exhibit $A\beta$ fibre-mediated EPSCs ($P < 0.01$, Fig. 3C). These $A\beta$ fibre-evoked EPSCs, all appear to be polysynaptic, defined in terms of frequency following and absence of latency jitter. Following SNI, a large percentage of neurones (19 out of 43; 44 %, $P < 0.01$) receive polysynaptic $A\delta$ fibre inputs, but only a small percentage display $A\beta$ fibre-mediated EPSCs (5 %). In addition, monosynaptic C fibre-mediated EPSCs are decreased after SNI. The percentage of neurones with monosynaptic $A\delta$ fibre EPSCs was reduced in all three peripheral nerve injury models.

The minimum stimulus intensity for eliciting A fibre-mediated EPSCs in slices from naive, SNT, CCI and SNI rats is shown in Fig. 4. In slices from SNT and CCI rats, mean EPSC threshold is significantly reduced compared to naive preparations. In contrast, mean EPSC threshold is significantly increased following SNI. The mean EPSC response threshold was $32 \pm 2 \mu\text{A}$ ($n = 81$) for naive, $22 \pm 2 \mu\text{A}$ ($n = 29$) for SNT, $23 \pm 2 \mu\text{A}$ ($n = 54$) for CCI and $49 \pm 4 \mu\text{A}$ ($n = 37$) for SNI.

DISCUSSION

The three nerve injury models examined in this study differ considerably. SNT involves transection of all the axons of the sciatic nerve. CCI produces both inflammation and compression and lesions in some, but not all, myelinated and unmyelinated axons of the sciatic nerve (Basbaum *et al.* 1991). SNI involves transection only of the axons of the tibial and common peroneal nerves, leaving the sural nerve intact (Decosterd & Woolf, 2000). In both the CCI and SNI models, there is behavioural pain hypersensitivity, in the former it is in the whole area of the damaged sciatic nerve territory (Bennett & Xie, 1988) and in the latter, it is restricted to the intact sural nerve territory (Decosterd & Woolf, 2000). In both SNI and CCI rats, but not SNT rats, there is a loss of GABA-mediated IPSCs in lamina II neurones (Moore *et al.* 2002).

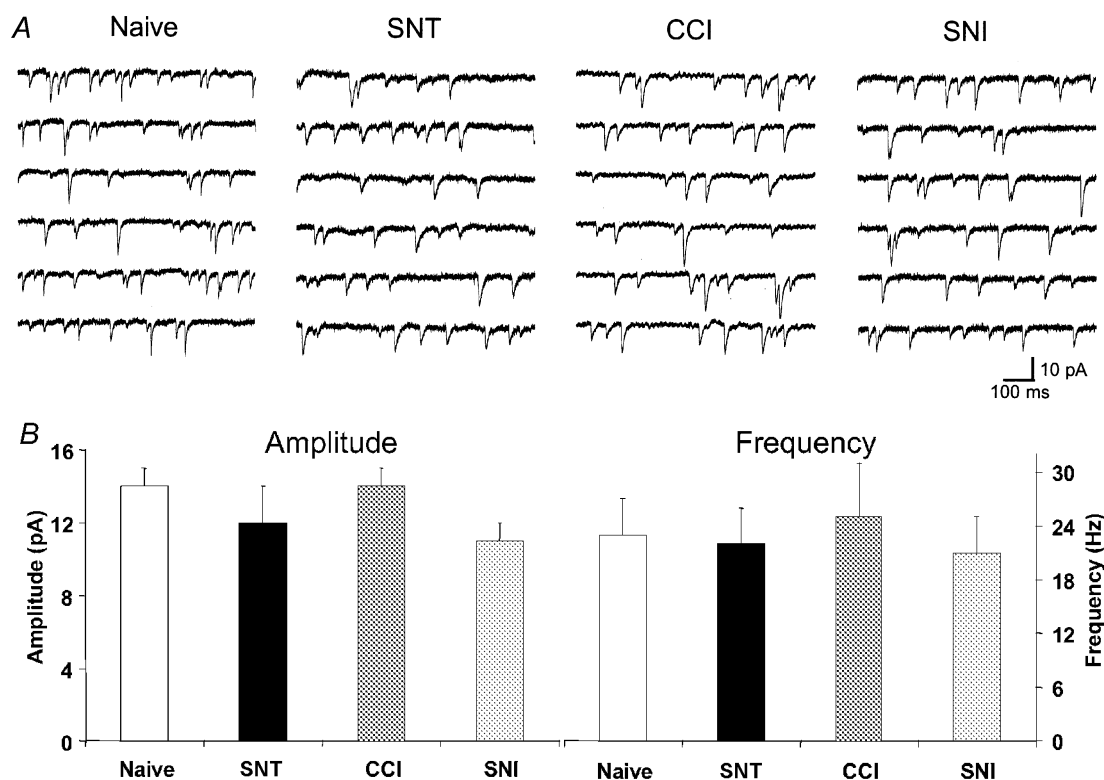


Figure 2. The amplitude and frequency of spontaneous EPSCs were unaffected by peripheral nerve injury

A, representative traces of spontaneous EPSCs. B, summary of the amplitude and frequency of sEPSCs recorded in slices from naive ($n = 14$), SNT ($n = 6$), CCI ($n = 9$) and SNI ($n = 15$) rats.

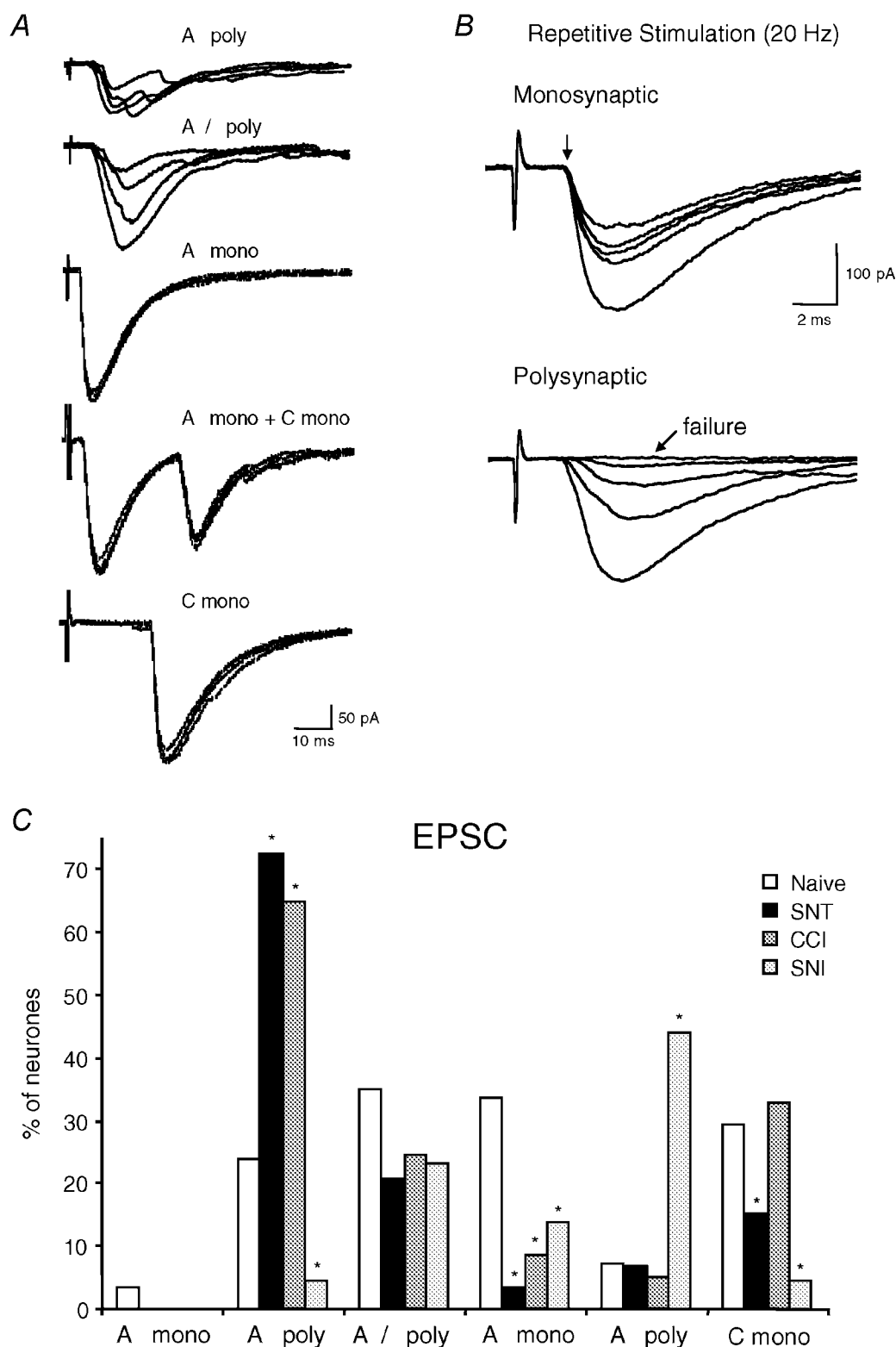


Figure 3. The distribution of EPSCs in lamina II neurones is altered following peripheral nerve injury

A representative EPSCs elicited by A β (10–25 μ A, 0.05 ms), A δ (25–50 μ A, 0.05 ms) and C (250–500 μ A, 0.5 ms) fibre stimulation. B fixed latencies and the absence of failures with high-frequency repetitive stimulation (20 Hz) defined A fibre inputs as monosynaptic. Conversely, variable latencies and the presence of failures with high-frequency stimulation defined inputs as polysynaptic. C, classification of EPSCs recorded in naive, SNT, CCI and SNI slices, $n = 83, 29, 57$ and 43 , respectively. Responses were grouped based upon response threshold, conduction velocity and response to high-frequency repetitive stimulation (20 Hz). * $P < 0.05$.

In this study, we utilised whole cell patch-clamp recordings from SG neurones to examine changes in excitatory synaptic inputs to the superficial dorsal horn. Our major findings are a shift in the response threshold

and a reduction in the frequency following capacity of A fibres. Following both SNT and CCI, there is the novel appearance of a significant population of SG neurones with A β fibre-mediated EPSCs, suggestive of substantial functional reorganisation of low-threshold inputs compared with the normal pattern in naive animals. In contrast, following SNI, the stimulus-response relationship of A fibre-evoked EPSCs is shifted to the right (i.e. a higher intensity stimulation is required to evoke A fibre-mediated EPSCs). In all three nerve injury models, no changes in the frequency or amplitude of spontaneous EPSCs are observed, suggesting that the presynaptic release machinery and postsynaptic AMPA/kainate receptors remain intact and functional.

Establishment of aberrant low-threshold synaptic connections

Almost all SG neurones are intrinsic inhibitory and excitatory interneurons, which integrate, modulate and filter primary afferent information before its transmission to the brain via projection neurones in lamina I, IV and V (Willis & Coggeshall, 1991). In naive animals there is a distinct laminar termination of primary afferent inputs, with C fibres terminating in lamina II, A δ fibres terminating in laminae I, II and V, and A β fibres terminating in laminae III, IV and V. Given the limited dendritic fields of the small SG interneurons this means that most are normally activated only by high-threshold A δ and C fibre inputs (Willis & Coggeshall, 1991).

Following SNT and CCI, the percentage of SG neurones with A β fibre-evoked EPSCs increased substantially from 24 to $\geq 65\%$. No change in dorsal root threshold or conduction velocity of A β , A δ and C fibres followed SNT or CCI. Therefore, it is unlikely that the recruitment of low-threshold EPSCs reflects changes in primary afferent fibre excitability. Surprisingly, a facilitation of A β fibre-mediated excitatory synaptic input was not detected in SNI rats, instead the mean response threshold actually increased.

A loss of monosynaptic A δ fibre EPSCs was observed in all three nerve injury models. A similar effect was reported in one previous study (Okamoto *et al.* 2001) that examined the effects of SNT on excitatory transmission in the superficial dorsal horn, but not in another (Kohama *et al.* 2000). The decrease in monosynaptic A δ fibre input correlates closely with the gain of A β fibre EPSCs in both the SNT and CCI models. Although a small decrease in afferent threshold following nerve injury could conceivably contribute to this shift in EPSC distribution, no such reduction follows SNT (Okamoto *et al.* 2001) or, as shown here, CCI and SNI. In addition to an alteration in threshold, there is also a marked shift from a mono- to a polysynaptic pattern of SG neurone activation after nerve injury. Such a change may reflect the recruitment of inputs via intervening synapses or a change in the frequency-following capacity of injured afferents (see below). The

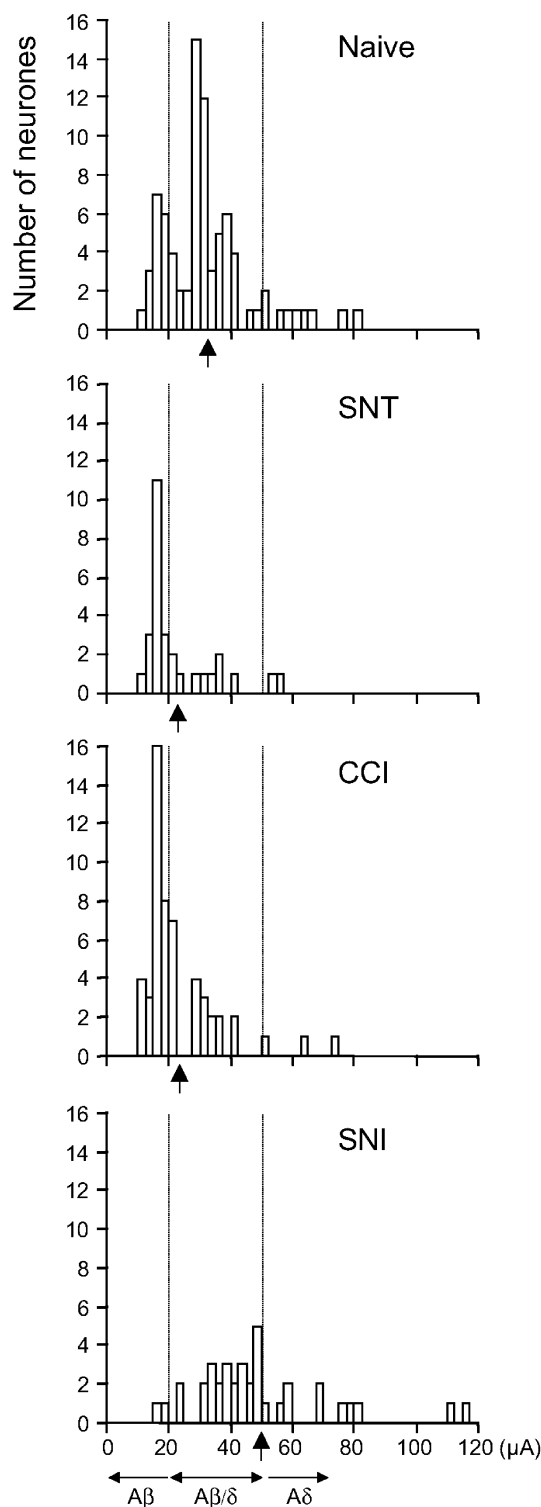


Figure 4. Response thresholds for A fibre-mediated EPSCs are altered by peripheral nerve injury

The mean A fibre response threshold was decreased following SNT ($P < 0.01$) and CCI ($P < 0.01$), but increased following SNI ($P < 0.01$). Arrows indicate mean thresholds.

most parsimonious explanation for the increased incidence of A β fibre EPSCs after CCI and SNT is a functional reorganization of excitatory inputs, not a change in peripheral activation. Following SNI, apart from the lack of recruitment of A β inputs, the A δ input to lamina II shifts from a largely mono- to a polysynaptic mode, suggesting that most monosynaptic inputs to lamina II neurones are lost, or that former monosynaptic A δ inputs are no longer capable of following high frequencies. It is unlikely that the difference between the SNI model and the CCI and SNT models is due to activation of injured or non-injured afferents or the degree of deafferentation. In this study, all recordings were made from the medial dorsal horn where injured afferents terminate in all three models.

Potential mechanisms of facilitated A β fibre input to the SG after peripheral nerve injury

Three possible mechanisms could operate to increase A β fibre inputs to SG neurones: recruitment of pre-existing silent synapses, sprouting of A β fibres from lamina I or III into lamina II and/or a facilitation of excitatory interneurones.

Silent synapses. Although there is no evidence for A β fibre central terminals in lamina II outer, the most dorsal tips of these afferents do terminate in the most ventral portion of lamina II inner (Shortland *et al.* 1989), and may make ineffective or silent synaptic contacts with ventrally directed dendrites of SG neurones. Silent NMDA synapses have been found in neonatal spinal cord, but are not present in naive adult lamina II neurones, and are found only rarely (4 %) following complete sciatic nerve transection (Baba *et al.* 2000). Therefore, silent NMDA synapses are unlikely to contribute to increased A β input following partial sciatic nerve injury. Whether there are silent AMPA/kainate synapses in lamina II neurones that can be recruited by insertion of AMPA receptors into the synaptic cleft is not known.

Sprouting of A fibres. A structural reorganization of low-threshold myelinated afferent terminals could also produce the altered synaptic connectivity that follows SNT and CCI. After peripheral nerve injury, A β fibres with the morphological appearance of hair follicle afferents sprout from the deep dorsal horn into the SG (Woolf *et al.* 1992; Koerber *et al.* 1994; Nakamura & Myers, 1999). In the SNI model, a similar change should occur for A β fibres in the injured common peroneal nerve, but the numbers of these relative to the other injury models will be less. The extent of sprouting A β fibres following nerve injury is controversial (Ma & Tian, 2001; Bao *et al.* 2002). Sprouting of A δ fibres from lamina I into the SG can also not be excluded (Ma & Tian, 2001).

After SNT, sprouted A β fibre terminals that make synaptic contact with lamina II neurones have been found (Koerber

et al. 1994, 1999). In one electrophysiological study, novel monosynaptic A β fibre-evoked responses were reported (Kohama *et al.* 2000), but in another, only polysynaptic A β inputs were found (Okamoto *et al.* 2001). We too only find a polysynaptic-like A β fibre activation of SG neurones after both SNT and CCI, as well as an increase in polysynaptic-like A δ fibre-evoked EPSCs after SNI. There are several potential explanations that may account for the increase in polysynaptic-like responses. First, A β fibre sprouting may be minimal and all input to SG neurones is via excitatory interneurones. Second, A β fibres may sprout and make new synaptic connections with SG neurones, but these connections may be nonfunctional. Finally, sprouted A β terminals may be functional, but may not follow strict monosynaptic criteria (no latency jitter and faithful following at high frequencies), due to branch point blockade resulting from atrophic changes in intraspinal terminals (Kapadia & LaMotte, 1987) and/or failure of synaptic release mechanisms during high-frequency stimulation. This is the explanation we favour.

Enhanced excitability. The increase in polysynaptic A fibre input to lamina II following partial or complete sciatic nerve injury might also reflect enhanced excitability of excitatory interneurones. A reduction in inhibition could produce such an increase in excitability following SNI or CCI where we have previously observed decreased inhibition (Moore *et al.* 2002). However, no loss of GABA-mediated IPSCs was observed after SNT (Moore *et al.* 2002), a model where there was a substantial increase in A β fibre input, suggesting disinhibition does not account for the recruitment of A β fibre inputs following injury. Alternatively, nerve injury might reduce action potential threshold or cause a direct increase in transmitter release from excitatory interneurones, thereby facilitating polysynaptic transmission. No change in mEPSC frequency was observed in any of the peripheral nerve injury models, arguing against such changes in interneurone excitability.

In conclusion, the present study demonstrates a recruitment of A β excitatory input to the superficial dorsal horn in the SNT and CCI, but not the SNI, models. These findings together with the previously demonstrated reduction in GABAergic inhibition following SNI and CCI, but not SNT (Moore *et al.* 2002), suggest that nerve injury models are not all equivalent in terms of the consequences on synaptic processing in the dorsal horn. The mechanisms underlying these differences and their contributions to changes in sensation now need to be established.

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